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EXPERIMENTAL DECOMPOSITION OF HUMIC ACID BY FUNGI

by

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The utilization of humic acid by micro-organisms has been reported by a number of authors, but in their work they have found that only a small proportion of the humic acid has been attacked, and usually concluded that it can serve only as a source of nitrogen. No previous evidence has been published showing the utilization of the non-nitrogenous portion of the humic acid or the quantitative destruction of the major portion of the molecule.

The humic acid used in this work was obtained from the B_1 horizon of a podzol at Delamere Forest, Cheshire, developed on glacial sands now covered by a plantation of *Pinus sylvestris*. It was extracted with 50 % v/v Lactic acid. The extract was passed through an ion exchange column containing amberlite 1R—120 resin and was then precipitated with hydrochloric acid and purified by dialysing first against dilute hydrochloric acid and then distilled water. The humic acid was then dried in vacuo at 45° C. An analysis gave the following percentage composition, C. 50; H. 3.9; O. 38.0; N. 0.4; Methoxyl 1.0; and Ash 1.5.

When the humic acid was used in culture media it was dissolved in 1 % K_3PO_4 and added to the basic media so that the final concentration was 0.12 %. The basic culture medium used had a pH of 6.8, and contained KH_2PO_4, 0.4; K_2HPO_4, 0.6; $MgSO_4$, 0.5; KCl, 0.5 gm. per litre, with trace elements and Fries' vitamin solution added, together with 200 μg per litre of vitamin B_{12}. Either 20 gm. sucrose or 2 gm. $NaNO_3$ were also added, to serve as a source of carbon or nitrogen respectively.

In order to isolate micro-organisms capable of decomposing humic acid, soil percolation columns were set up using the apparatus described by Neguib (1). They contained soil or sterile sand and were perfused with the basic mineral solution to which humic acid was added as either the sole source of carbon, or nitrogen. Similar control columns were set up containing 1 % K_3PO_4 solution but no humic acid. All columns were inoculated with a mixed suspension from a fertile clay soil and composting cow manure. The experiment was continued for some months by inoculating fresh columns with soil from the previous corresponding column every eight weeks. At each transfer the microbial flora of the soil and the perfusate were examined using soil crumb and dilution plates, and direct observation. The basic medium in the plates used for the isolations was the same as for the perfusate but with the addition of 2 % agar. Humic acid was added to all plates, making the medium dark brown in colour. A number of the fungal and bacterial colonies arising from the soil crumbs and in the dilution plates produced a decolourised zone in the humic acid agar. In previous work it had been found that the production of decolourised zones could be caused by the accumulation of the humic acid in or around the hyphae (2, 3). Some *Penicillia* spp. were particularly active in causing this type of decolourisation. Although many of these zones were caused by accumulation of the humic acid, a few fungi were isolated which gave a decolourised zone but showed no apparent evidence of accumulation. Two of the most active, were selected for further study in pure culture. One of these formed a brilliant yellow colony with simple branched conidiophores producing chains of conidia. It was provisionally referred to Spicaria (4) but subsequently characteristic perithecia and ascospores, as well as a few biverticillate asymmetric conidiosphores were formed and the fungus was identified as a strain of *Penicillium luteum*. The other fungus selected for study produced white mycelium with clamp connections and the characteristic growth form of a basidiomycete. Small ill-formed fruiting structures with a poroid hymenium were formed in culture. Comparison with known cultures of *Polystictus versicolor* led to the isolate being identified provisionally as *Polystictus* sp.

The *Polystictus* and the *Penicillium* were both isolated from perfusion tubes in which the medium contained sucrose but no nitrogen source other than the humic acid. Initial decomposition experiments were therefore carried out using a similar medium. Penicillin flasks were prepared containing 500 ml. of medium to which 0.2 % Agar, and 0.039 % humic acid had been added. Soil perfusion columns were also prepared containing sterile soil and were perfused with the medium with 0.12 % humic acid added. Decomposition was confirmed by extracting and weighing the residual humic acid from the medium, and from the mycelium or soil at the end of the experiment. Table 1 shows the results of one set of such experiments.

TABLE 1. *Recovery of humic acid from pure cultured experiments after eight weeks.*

	Soil Percolation columns		Penicillin flasks	
	mg humic acid			
	Added	Recovered	Added	Recovered
Control	81	77	195	210
Pen. luteum	66	44	195	162
Polystictus sp.	77	44	195	70

It can be seen that in the penicillin flasks approximately 70 % of the humic acid had been decomposed by *Polystictus*. *Penicillium luteum* is less active, decomposing only about 30 % of the humic acid during the same period. In agar plate cultures (9 cm. Petri dish) containing humic acid *Polystictus* caused complete decolourisation of the medium in 3—4 weeks whereas with *P. luteum* the decolourisation was primarily around and below the restrictedly growing colony.

A number of workers have reported the bleaching of malt agar, (5, 6) and the decolourisation of various organic dyes by basidiomycetes and in view of the possible similarity between these activities and the decolourisation of humic acid, the fungi isolated were examined for their activity on these other substrates. The ability to decolourise dyes such as neutral red has also been shown to be correlated with the ability to cause white rots (7) and such fungi were therefore also tested on humic acid agar and their activity compared with that on neutral red and malt agar. The reaction of these same fungi was also tested on other types of humic acid obtained from various sources, including a saccharo-humic acid prepared by treating sucrose with hydrochloric acid. The tests were carried out by inoculating at the centre of the agar plates and observing the decolourised area which appeared round the developing colony. A selection of the results are shown in table 2.

TABLE 2. *Decolourisation of various organic substrates by fungi.*

—	Delamere Humic acid	Neutral Red	Malt Agar	Saccharo-Humic acid	Lights' Co. Humic acid	Anthraxylic acid (Prof. Gillet)	Oxidised Coal Extract (Dr. Mills)
Penicillium luteum	(+)	—	—	(+)	(+)	—	(+)
Polystictus sp.	+	+	+	+	+	(+)	(+)
P. versicolor	+	+	+	+	+	(+)	+
P. adustus	—	—	—	—	Not tested		
Hypholoma fasciculare	+	+	+	+	+	(+)	—

+ Medium decolourised.
(+) Medium partially decolourised.

It can be seen that Basidiomycetes which are able to decompose humic acid, can, in most cases also decolourise the other substrates and also neutral red. Other Basidiomycetes which also decolourise humic acid, include *Ganoderma lucidum*, and *Trametes rubexens*, as well as some which can partially decolourise it, including *Collybia velutipes*, *Daedalia unicolor*. The pyrenomycete *Ustulina zonata* can also decolourise humic acid partially.

Law (7) showed that *Polystictus versicolor* was able to decolourise a wide range of dyes of very different chemical constitution. The ability of our organisms to decompose a range of humic acid preparations therefore does not necessarily indicate a similarity of chemical structure. Our tentative proposal is that a wide spectrum oxidase is involved in the breakdown of the humic materials.

The correlation between these different activities of the wood rotting fungi suggested that the activity might be due to the laccase enzyme which is produced in quantity by such fungi. A medium for the production of high yields of laccase by Basidiomycete cultures, has been developed by Fahraeus (8) and cultures were therefore set up in his medium Bf-2 (9), with or without the addition of the inducer (2,5—xylidine) but with humic acid also added. The medium was found to be excellent for the growth of *Polystictus*, but the decolourisation was very slight. Tests confirmed that abundant laccase was produced. Active laccase preparations from Polystictus were tried on humic acid but no decolourisation was detected. ,

Fahraeus' medium Bf-2 without inducer was then used in 5 litre aspirators with bubble aeration in order to obtain good rapid growth of *Polystictus* in pellet form. The pellets were collected by centrifugation and were resuspended in 250 ml. conical flasks containing 50 ml. of basic mineral solution with both carbon and nitrogen source added, together with 0.06 % humic acid. The flasks were incubated at 25° C on a shaking machine. Humic acid concentration was determined colorimetrically on an EEL colorimeter with blue filter, OBIO, after suitable dilution with Sørensen's phosphate buffer pH 7.0. Optical density readings were linear over a range of humic acid concentrations from 0.001 % to 0.006 %. Decolourisation was almost complete at 6 days by this method and allowance for absorbtion by the mycelium was made by extracting pellets with 1 % K_3PO_4 at the end of the experiment. The results of one experiment are shown in table 3.

TABLE 3. *Decomposition of humic acid by pellets of Polystictus after 6 days in shakeflask culture.*

mg humic acid			
Added	Residual in medium	Extracted from mycelium	Decomposed
31.5	5.9	2.7	22.9

It can be seen that 81 % of the original humic acid had disappeared from the medium of which only 9 % would be accounted for by absorbtion on the mycelium leaving a total decomposition of 72 %. The medium remaining at the end of the experiment was pale yellow in colour, it gave a slow precipitation of a small amount of humic acid. Consideration was given to the possibility that the humic acid had been reduced to a leucoform. The medium at the end of the experiment did not darken on exposure to air even when made strongly alkaline, nor would the usual oxidising agents produce any darkening.

We therefore conclude that a genuine decomposition of the humic acid is here taking place and that the whole molecule is subject to the attack.

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SUMMARY

By the use of a soil perfusion enrichment technique two fungi, *Penicillium luteum* and *Polystictus* sp. capable of decomposing soil humic acid were isolated. Evidence is presented to show that *P. luteum* was able to decompose 30 % and *Polystictus* 70 % of the humic acid added to the cultures. Examination of a range of basidiomycetes known to cause white rots showed that a number of these were also able to decompose humic acid. Some of these were also capable of decomposing a commercial sample of humic acid from coal, (Lights' Co. Ltd.), Gillet's anthraxylic acid and saccharo-humic acid. The apparently related ability to decolourise malt agar and organic dyes was also examined.

RÉSUMÉ

Deux champignons, capables de décomposer l'acide humique du sol, *Penicillium luteum* et *Polystictus* sp., ont pu être isolés grâce à une technique d'enrichissement par perfusion. Il a été montré que *P. luteum* pouvait décomposer 30% de l'acide humique ajouté aux cultures, *Polystictus* 70 %. L'examen d'une série de Basidiomycètes, dont on savait qu'ils pouvaient causer la pourriture blanche, montrait qu'un certain nombre d'entr'eux décomposaient également l'acide humique. Certains décomposaient aussi un acide humique commercial, extrait de charbon (Lights 'Co. Ltd.) l'acide anthraxylique de Gillet et l'acide saccharo-humique. La décoloration du milieu 'malt-agar-colorants organiques', effet vraisemblablement en rapport avec ceux décrits plus haut, a également été examiné.

ZUSAMMENFASSUNG

Mittels einer Anreicherungstechnik durch Bodenberieselung konnten zwei Schimmelarten, *Penicillium luteum* und *Polystictus* sp., welche die Huminsäure des Bodens zu zersetzen vermögen, isoliert werden. Es wird gezeigt dass *P. luteum* im Stande war, 30 % der Huminsäure, den Kulturen zugefügt, zu zersetzen; *Polystictus* sogar 70 %. Untersuchung einer Reihe von Basidiomyceten, von welchen bekannt ist dass sie 'weisse Fäulnis' verursachen, ergab, dass eine Anzahl derer ebenfalls Huminsäure zu zersetzen vermag. Manche von diesen waren auch im Stande eine Handelsprobe von Huminsäure aus Kohle (von Lights' Co. Ltd.), Gillet's Anthraxylsäure und Saccharohuminsäure zu zersetzen. Das augenscheinlich damit in Beziehung stehende Vermögen, Malzagar und organische Farbstoffe zu entfärben, wurde mit untersucht.

DISCUSSION

H. DEUEL: How does the humic acid influence the growth of the microorganisms?

P. LATTER: There is some evidence that growth of these two fungi is increased in media deficient in nitrogen. The humic acid used here contains 0.4 % N and this would be released during decomposition. In complete culture media no effect on growth has been observed.

M. ALEXANDER: Can the technique of simultaneous adaption be used to study the pathway of decomposition of humic acid?

P. LATTER: We have not used this method up to now. Evidence obtained so far has shown that there is no lag in the commencement of decomposition in fully developed cultures, so that the methods of simultaneous adaption may not be applicable in this case.

W. FLAIG: Has the methoxyl content of the humic acid changes after treatment with phenoloxydases?

P. LATTER: The methoxyl content of the extracted humic was unchanged. We have not used phenoloxydases isolated from fungi.